Olig2 and Ngn2 function in opposition to modulate gene expression in motor neuron progenitor cells

Soo-Kyung Lee,[1] Bora Lee,[1] Esmeralda C. Ruiz, and Samuel L. Pfaff[2]

Gene Expression Laboratory, The Salk Institute for Biological Studies, La Jolla, California 92037, USA

Spinal motor neurons and oligodendrocytes are generated sequentially from a common pool of progenitors termed pMN cells. Olig2 is a bHLH-class transcription factor in pMN cells, but it has remained unclear how its transcriptional activity is modulated to first produce motor neurons and then oligodendrocytes. Previous studies have shown that Olig2 primes pMN cells to become motor neurons by triggering the expression of Ngn2 and Lhx3. Here we show that Olig2 also antagonizes the premature expression of post-mitotic motor neuron genes in pMN cells. This blockade is counteracted by Ngn2, which accumulates heterogeneously in pMN cells, thereby releasing a subset of the progenitors to differentiate and activate expression of post-mitotic motor neuron genes. The antagonistic relationship between Ngn2 and Olig2 is mediated by protein interactions that squelch activity as well as competition for shared DNA-binding sites. Our data support a model in which the Olig2/Ngn2 ratio in progenitor cells serves as a gate for timing proper gene expression during the development of pMN cells: Olig2^{high} maintains the pMN state, thereby holding cells in reserve for oligodendrocyte generation, whereas Ngn2^{high} favors the conversion of pMN cells into post-mitotic motor neurons.

[*Keywords*: Motor neuron; oligodendrocyte; development; basic helix–loop–helix (bHLH); neurogenin (Ngn); Olig]

Supplemental material is available at http://www.genesdev.org.

Received September 1, 2004; revised version accepted November 17, 2004.

Neuroepithelial cells within the developing CNS have a remarkable ability to produce different types of neuronal and glial progeny. The fate of these CNS founder cells is influenced by numerous extracellular signals during embryonic development (Tanabe and Jessell 1996; Jessell 2000), leading to the regional specification of progenitor pools assigned to produce different cell types (Briscoe and Ericson 2001). An additional mechanism for expanding cellular diversity in the CNS is the sequential generation of different cell types from a common precursor cell population. For example, lineally related cells in the neocortex sequentially generate cortical neurons that acquire different laminar fates (McConnell 1995), and retinal precursors produce different classes of retinal neurons in a serial fashion (Cepko 1999). Likewise, pMN progenitor cells in the spinal cord initially produce motor neurons, but switch later in development to forming oligodendrocytes (Richardson et al. 2000; Rowitch et al. 2002). Thus, the daughters of pMN cells select whether to retain their pMN identity, differentiate into motor neurons, or become oligodendrocytes—representing choices associated with dramatically different patterns of gene expression. Although regulatory mechanisms involving cross-repressive and derepressive interactions between transcription factors have begun to be elucidated in the developing spinal cord (Briscoe et al. 2000; Muhr et al. 2001; Lee et al. 2004), the mechanisms that underlie the decisive timing of regulation of genes within a progenitor population as it produces different cell types remain poorly understood.

Motor neurons and oligodendrocytes perform different functions in the spinal cord: motor neurons extend axons from the spinal cord, synapse with muscles, and control movements with the neurotransmitter acetylcholine; whereas oligodendrocytes are glial cells that myelinate the axons of neurons within the CNS. Nevertheless, the development of these two divergent cell types is closely linked. The conversion of unspecified neuroepithelial cells in the ventral neural tube to pMN precursor cells is driven by Sonic hedgehog (Shh) from the notochord and floor plate and retinoic acid (RA) likely produced by the paraxial mesoderm (Ericson et al. 1997; Novitch et al. 2003). These extracellular signals are read out by emergent pMN cells, leading to the expression of a unique constellation of homeodomain transcription factors in-

[1]**Present address: Department of Molecular and Cellular Biology, Huffington Center on Aging, Baylor College of Medicine, One Baylor Plaza, Room 132C, Houston, TX 77030, USA.**
[2]**Corresponding author.**
E-MAIL pfaff@salk.edu; FAX (858) 453-2573.
Article and publication are at http://www.genesdev.org/cgi/doi/10.1101/gad.1257105.

cluding Nkx6.1, Nkx6.2, Pax6, Mnr2 (chick-specific gene), and Lhx3 (chick Lim3) that distinguish motor neuron/oligodendrocyte precursors from those for other cell types (Jessell 2000; Briscoe and Ericson 2001). Interestingly, as pMN cells switch from producing motor neurons to generating oligodendrocytes, Nkx2.2 is up-regulated, thereby altering their transcription factor profile (Qi et al. 2001; Zhou et al. 2001; Sun et al. 2003; Liu and Rao 2004). Thus, motor neurons and oligodendrocytes are both derived from pMN cells, but the transcriptional codes within this cell population are not static during development.

In addition to homeodomain proteins, recent studies have demonstrated important roles for the basic helix–loop–helix (bHLH)-class transcription factors Ngn1/2 and Olig1/2 in motor neuron and oligodendrocyte development (Scardigli et al. 2001; Lu et al. 2002; Zhou and Anderson 2002). Olig1/2 (chickens appear to lack Olig1) are expressed during an extensive portion of pMN cell development encompassing the period when both motor neurons and oligodendrocytes develop (Lu et al. 2000; Zhou et al. 2000; Mizuguchi et al. 2001; Novitch et al. 2001; Zhou et al. 2001). *Olig1/2* mutant mice lack spinal motor neurons and oligodendrocytes due to the conversion of pMN cells into progenitors for V2 interneurons and astrocytes (Lu et al. 2002; Zhou and Anderson 2002). Ectopic expression of Olig2 in the dorsal neural tube of chick embryos drives the differentiation of neuroepithelial cells into neurons, which likely occurs by activating expression of the neurogenic bHLH transcription factor Ngn2 (Novitch et al. 2001; Lu et al. 2002; Zhou and Anderson 2002). Thus, both Olig2 and Ngn2 are expressed by pMN cells, and functional studies have shown that both are involved in motor neuron differentiation (Mizuguchi et al. 2001; Novitch et al. 2001; Scardigli et al. 2001; Lu et al. 2002; Zhou and Anderson 2002; Lee and Pfaff 2003). Based on the regulatory interactions of Olig1/2 with the other factors involved in motor neuron specification, these bHLH factors occupy a key nodal point in the transcriptional pathway controlling pMN cell fate—acting to coordinate the expression of genes for neuronal subtype identity with those for neurogenesis (Novitch et al. 2001). Coexpression of Olig2 and Ngn2 in pMN cells occurs during the period of motor neuron formation; however, at later stages when oligodendrocytes are produced, Ngn2 becomes down-regulated, which is likely an obligatory requirement since it directly antagonizes glial cell differentiation (Sun et al. 2001; Zhou et al. 2001).

Although Olig1/2 contribute to the regulation of *Ngn2* (Novitch et al. 2001; Zhou and Anderson 2002), the functional relationship between these genes has been difficult to decipher (Marquardt and Pfaff 2001). Coexpression of Ngn2 with Olig2 in the dorsal neural tube of chick embryos has been found to enhance the ability of Olig2 to trigger the ectopic formation of motor neurons (Mizuguchi et al. 2001). This seems consistent with the finding that pMN cells express both Olig2 and Ngn2 during the period in which their progeny become motor neurons (Mizuguchi et al. 2001). Nevertheless, these findings have been hard to reconcile with the observation that Olig2 misexpression is sufficient on its own to induce transcription of the endogenous *Ngn2* gene in dorsal-neural tube cells (Novitch et al. 2001). A complicating issue that also remains poorly understood is the restricted ability of Olig2 to promote ectopic motor neuron formation. Unlike other transcription factors such as MNR2, Nkx6.1, and Isl1 plus Lhx3, which trigger motor neuron differentiation along the entire dorsal–ventral axis of the neural tube when ectopically expressed (Tanabe et al. 1998; Briscoe et al. 2000; Thaler et al. 2002), ectopic motor neuron formation triggered by Olig2 is restricted to a more isolated region of the neural tube just dorsal to the endogenous motor neuron population (pV2 cells) (Mizuguchi et al. 2001; Novitch et al. 2001). Interestingly, pMN and pV2 cells share the expression of Ngn2 and Lhx3 (Mizuguchi et al. 2001; Scardigli et al. 2001; Thaler et al. 2002), and the presence of these factors might facilitate the ability of Olig2 to drive motor neuron formation (Mizuguchi et al. 2001).

As pMN cells commit to become post-mitotic motor neurons, numerous changes in gene expression occur. Ngn2 is replaced by the proneural bHLH factors NeuroM and then NeuroD, which have similar functions to that of Ngn2 in the way they contribute to motor neuron specification (Lee and Pfaff 2003; Lee et al. 2004). In addition, the LIM homeodomain factors Isl1 and Lhx3/4 become coexpressed at this transition point, allowing a heteromeric ternary complex to form with the nuclear LIM interactor protein NLI (Ldb, Clim) (Thaler et al. 2002). This homeodomain complex synergizes with the proneural bHLH factors present in these cells and regulates the expression of genes such as *Hb9* involved in the post-mitotic development of motor neurons (Arber et al. 1999; Thaler et al. 1999; Lee and Pfaff 2003). In contrast to the bHLH factors NeuroM/D, however, Olig1/2 are abruptly extinguished from developing motor neurons as they develop from pMN cells (Lu et al. 2000; Zhou et al. 2000; Mizuguchi et al. 2001; Novitch et al. 2001). Thus, while Ngn2 and Olig2 are both bHLH factors expressed by multipotential precursors, Ngn2 is extinguished from the progeny that become oligodendrocytes and Olig2 is down-regulated in the daughters that become motor neurons.

To better understand how the activity of Olig2 is modulated to control gene expression for motor neuron formation at early stages of pMN development, we investigated the functional relationship between Olig2 and Ngn2. We found that individual pMN cells contain heterogeneous levels of Olig2 and Ngn2 protein during the period of motor neuron generation. The transcriptional activity of Ngn2 mediated by binding to E-box elements is found to be antagonized by Olig2. The mechanistic basis for this antagonism appears to be through competitive binding for E-box elements as well as squelching the A class bHLH E47 dimerization partner away from Ngn2. Functional studies indicate that Olig2 down-regulation is an obligatory requirement for releasing pMN cells from their inhibitory block on post-mitotic motor neuron formation. Thus, Ngn2 and Olig2 appear to have

cross-regulatory interactions and cross-functional interactions that control the fate of pMN cells.

Results

pMN cells express Olig2 and Ngn2 heterogeneously

To investigate the relationship between Olig2 and Ngn2 in motor neuron progenitor cells (pMNs), we first examined the expression of these two proteins in the developing chick neural tube using immunocytochemistry. At Hamburger Hamilton (HH) stage 17 (Hamburger and Hamilton 1951), a peak period of motor neuron generation, Olig2 and Ngn2 are detected in the nuclei of pMN cells (Fig. 1A,B; Mizuguchi et al. 2001; Novitch et al. 2001; Scardigli et al. 2001). Ngn2 is also found in non-pMN cells located more dorsally in the progenitor cells for V2 interneurons, as well as other classes of differentiating neurons in the dorsal neural tube (Scardigli et al. 2001). Within the pMN domain the overlap between Olig2 and Ngn2 was extensive; however, relative levels of the proteins within individual nuclei varied (Fig. 1C). This heterogeneity among cells within the pMN domain became even more apparent at HH stage 21 when motor neuron differentiation ceases. At this point we found that Ngn2 was down-regulated in the pMN cell domain and Olig2 labeling became more prominent (Fig. 1D–F).

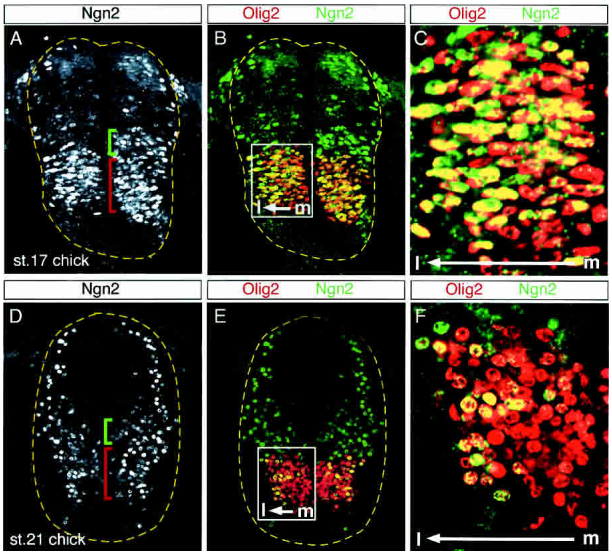

Figure 1. Immunohistochemical analysis of bHLH transcription factor expression in the developing chick neural tube. (*A*,*B*) Olig2 and Ngn2 are coexpressed in chick embryo pMN cells (red bracket, *A*) at HH stage 17. Ngn2 is also expressed in Olig2$^-$ pV2 cells (green bracket, *A*) and dorsally located post-mitotic cells. (*C*) Enlargement of the boxed area in *B* reveals that the relative levels of Ngn2 and Olig2 vary within individual pMN cells. This results in a mix of red (Olig2^{high}) and yellow (Olig2$^+$/Ngn2$^+$) labeling. (*D–E*) At HH stage 21, Olig2$^+$/Ngn2$^+$ cells are located at the subventricular zone where motor neurons emerge. In contrast, Olig2$^+$/Ngn2$^-$ cells are predominantly found medially in the proliferative ventricular zone. (*F*) Enlargement of the boxed area in *E* reveals reduced levels of Ngn2 in the Olig2$^+$ pMN cells at HH stage 21. The medial (m) to lateral (l) orientation of the ventricular zone is shown in *B*, *C*, *E*, and *F*.

Olig2 antagonizes the neurogenic activity of Ngn2

Ngn2 is a neurogenic bHLH transcription factor that promotes cell cycle exit and the up-regulation of pan-neuronal genes in neuroepithelial cells. As expected, transfection of mouse embryonic P19 cells with an Ngn2-expression construct activates expression of the pan-neuronal genes β-tubulin and neurofilament (Fig. 2A,B,E; Farah et al. 2000; Lee and Pfaff 2003; data not shown). In addition, the shape of Ngn2-expressing P19 cells changes from an epithelial to a neuronal morphology with neurite processes extending from the cell bodies. Olig2 is found to promote neurogenesis when ectopically expressed in the chick embryo neural tube (Mizuguchi et al. 2001; Novitch et al. 2001). In P19 cells, however, we found that Olig2 was much less efficient than Ngn2 at activating neuronal gene expression and driving the morphological conversion of these cultured cells into neurons (Fig. 2C–E). Thus, Olig2 has weaker neurogenic activity than Ngn2 in P19 cells.

The apparent differences in neurogenic activity of Olig2 compared to Ngn2 prompted us to test whether these factors could function additively or perhaps synergize to promote neuronal differentiation. We cotransfected Ngn2 and Olig2 expression constructs into P19 cells and monitored neuronal differentiation. While 50% of the cells transfected with Ngn2 expressed the neuronal marker β-tubulin within 24 h, no cells were labeled following Olig2 transfection (Fig. 2E). The addition of Ngn2 and Olig2 failed to enhance neurogenesis, and instead neuronal differentiation was suppressed from the levels obtained with Ngn2 alone (Fig. 2E). Thus, no evidence was found for cooperation between Ngn2 and Olig2 in neurogenesis, and instead Olig2 seemed to interfere with the ability of Ngn2 to promote neuronal differentiation.

Misexpression and loss-of-function studies have shown that Olig proteins activate *Ngn2* expression (Novitch et al. 2001; Lu et al. 2002; Zhou and Anderson 2002). This regulatory link combined with the functional interactions between Olig2 and Ngn2 in P19 cells hinted that a complex feedback relationship might exist between these transcription factors in the spinal cord. This prompted us to compare the neurogenic activity of Ngn2 alone to that of Olig2, an inducer of Ngn2, in electroporated chick embryo neural tube cells. Under similar experimental conditions we found that Ngn2 was more efficient than Olig2 at triggering expression of the neuronal marker TuJ (Fig. 2F–K). Normally TuJ1$^+$ cells are located laterally in the neural tube (see nonelectroporated side of neural tube) (Fig. 2F,I). In contrast, Ngn2 activated TuJ1 expression across the entire medial–lateral axis of the neural tube (Fig. 2H). Olig2 failed to activate TuJ1 medially, and most Olig2-expressing cells lacked the neuronal marker (Fig. 2J,K). These findings indicated that the probability of neuronal differentiation was greater for cells with Ngn2 than for cells with Olig2.

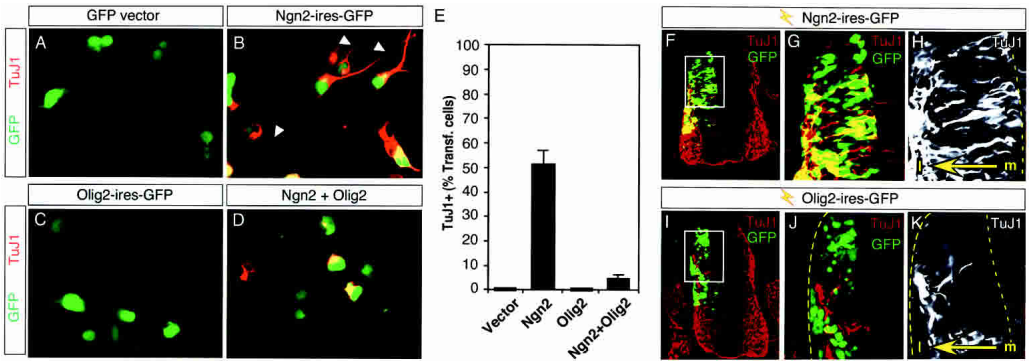

Figure 2. Olig2 attenuates the neurogenic activity of Ngn2. (*A–D*) Neuronal differentiation assays in transfected P19 cells cultured 3 d using anti-β tubulin neuronal marker antibody TuJ1 (red). (*A*) P19 cells transfected with GFP vector control lack TuJ1$^+$ neurons. (*B*) Ngn2 triggers β-tubulin expression (red) in most transfected cells (green), and induces the formation of neurite processes (arrowheads). (*C*) Olig2 failed to drive neuronal differentiation in P19 cells. (*D*) Olig2 markedly reduced the ability of Ngn2 to induce β-tubulin expression and prevented the formation of neurite processes. (*E*) The neurogenic activity of Ngn2 and Olig2 was determined by calculating the percentage of transfected cells (GFP$^+$) labeled by TuJ1 after 1 d in culture. (*F–K*) TuJ1 neuronal marker induction in chick embryo neural tubes 43 h post-electroporation. (*F–H*) Ngn2 induces ectopic and precocious TuJ1 neuronal marker expression. Many Ngn2$^+$ cells coexpress TuJ1 (yellow) and TuJ1 labeling of neurons is detected across the medial (m) lateral (l) axis of the neural tube. The box in *F* corresponds to approximate regions shown in *G* and *H*. (*I–K*) Olig2 is less efficient than Ngn2 at driving neuronal differentiation. In contrast to Ngn2, Olig2$^+$ cells rarely express TuJ1 and fewer TuJ1$^+$ cells are detected across the m–l axis. The boxed area in *I* corresponds to images in *J* and *K*.

Constitutive Olig2 expression interferes with motor neuron generation

The misexpression of Olig2 triggers ectopic motor neuron generation; however, it is relatively inefficient, and its activity is restricted to the V2 interneuron domain just dorsal to motor neurons (Mizuguchi et al. 2001; Novitch et al. 2001). To examine the function of Olig2 in motor neuron specification, we began by using CMV-based promoters to drive constitutive expression of Olig2–ires–GFP in electroporated chick embryo neural tube progenitor cells. The pattern of GFP expression was then correlated with the effect of Olig2 on motor neuron generation.

Two distinct phenotypes were observed in the neural tube following Olig2 misexpression that appeared to depend on the ratio of electroporated to nonelectroporated cells. Embryos in which forced expression of Olig2 was achieved in a majority of neural tube cells unexpectedly generated reduced numbers of post-mitotic motor neurons marked by the Isl1 protein (Fig. 3A–C). We considered the possibility that this was a nonspecific effect; however, these embryos still generated ectopic motor neurons in the V2 domain, which supports the argument against a general block in motor neuron development (Fig. 3C). Embryos with a mosaic distribution of Olig2 expression generated motor neurons in normal numbers from the pMN domain; however, we noted a selective bias in the distribution of Olig2-electroporated cells marked by GFP. A conspicuous gap in the GFP labeling around the endogenous Isl1$^+$ motor neuron domain indicated that Olig2-expressing cells were selected against becoming motor neurons (Fig. 3D–F). Together these findings suggest that the maintenance of high Olig2 levels interferes with the proper generation of post-mitotic motor neurons, whereas motor neurons generated ectopically in the V2 domain appeared resistant to this effect.

The inhibitory effects of Olig2 on motor neuron differentiation were counter to our expectations. Therefore, we considered the possibility that this might represent a "premature differentiation" phenotype caused indirectly by driving neuroepithelial cells into a terminal pathway of neuronal differentiation. In principle, this could reduce the overall number of motor neurons by rapidly depleting the progenitor cell reservoir. Although the conditions we used to express Olig2 appeared to be relatively neutral, neither promoting nor inhibiting neurogenesis, we attempted to circumvent this potential complication by testing the activity of Olig2 in cells artificially forced to become motor neurons through the ectopic expression of Isl1 and Lhx3. Inclusion of Ngn2 with the LIM factors has been shown to enhance motor neuron formation by approximately sixfold (Fig. 3G; Lee and Pfaff 2003). Because Olig2 activates Ngn2 expression (Novitch et al. 2001), we expected that coexpression of Olig2 together with Isl1 and Lhx3 would likewise enhance the activity of the LIM factors. Nevertheless, we found that Olig2 was unable to enhance the activity of Isl1 and Lhx3 (Fig. 3G).

Next we examined the functional relationship between Olig2 and the LIM factors using P19 cell transfections and the Hb9 marker to monitor motor neuron differentiation. Although Olig2 is able to promote ectopic motor neuron formation in the neural tube (see Fig. 3C,F; Mizuguchi et al. 2001; Novitch et al. 2001), it was unable to specify the motor neuron fate in P19 cells (Fig. 3H). Moreover, Olig2 lacked the ability to synergize with Isl1 and Lhx3 to trigger motor neuron differentiation, whereas Ngn2 cooperated strongly with the LIM proteins (Fig. 3H; Lee and Pfaff 2003). Our results demonstrate that

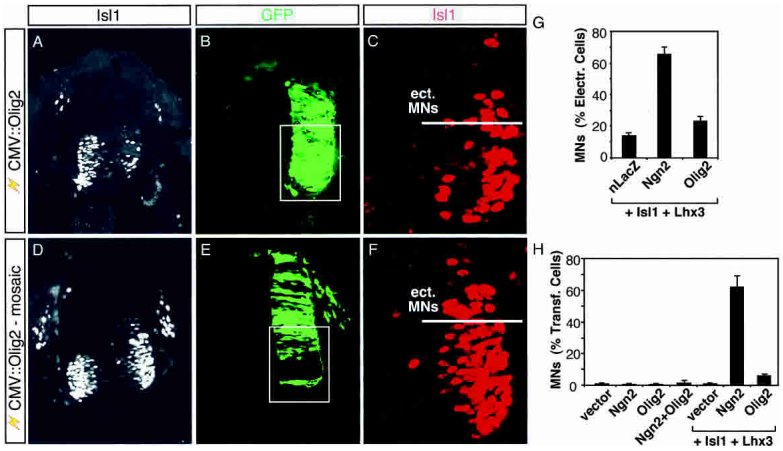

Figure 3. Constitutive expression of Olig2 is detrimental to motor neuron generation. (*A–F*) Immunocytochemical analysis of motor neuron differentiation monitored using the Isl1 marker of these cells in HH stage 20 chick embryos electroporated with CMV–Olig2–ires–GFP. (*A–C*) In embryos with a high percentage of electroporated (GFP$^+$) cells, Isl1 levels were markedly reduced in the endogenous motor neuron domain concomitant with development of ectopic motor neurons (ect. MNs) in the V2 domain. The box in *B* corresponds to the image in *C*. (*D–F*) Electroporated embryos with a mosaic distribution of Olig2 marked by more scattered GFP labeling exhibited a selective bias in the distribution of Olig2-expressing cells. Because of apparent sorting, motor neurons developed normally but were derived from cells lacking constitutive Olig2. Again, ectopic motor neurons were detected (ect. MNs). The box in *E* corresponds to image in *F*. (*G*) Motor neuron differentiation analysis (Hb9$^+$ cells) in HH stage 22 chick embryos electroporated with the indicated LIM and bHLH constructs. Coelectroporation of Ngn2 with Isl1 and Lhx3 potentiates motor neuron generation, whereas Olig2 lacks this stimulatory effect. (*H*) Motor neuron induction assays in P19 cells. Olig2 expression alone or in combination with Ngn2 failed to trigger motor neuron differentiation. Unlike Ngn2, Olig2 is unable to synergize with Isl1 and Lhx3 to specify motor neurons.

Olig2 and Ngn2 differ in their ability to synergize with LIM homeodomain factors to specify motor neurons.

Olig2 represses the Hb9 *motor neuron gene*

During spinal cord development Olig2 is down-regulated in differentiating motor neurons, but it is maintained in pMN progenitor cells and differentiating oligodendrocytes (Fig. 4A,B; Lu et al. 2000; Zhou et al. 2000; Mizuguchi et al. 2001; Novitch et al. 2001; Zhou et al. 2001). The negative effect of Olig2 on endogenous motor neuron differentiation when expressed constitutively, combined with the finding that Olig2 is excluded from motor neurons as they differentiate, prompted us to ask whether Olig2 affects post-mitotic motor neuron gene expression. To test this we coexpressed Olig2 with regulatory elements from the *Hb9* gene (*Hb9::GFP*) that direct gene expression to post-mitotic motor neurons (Lee et al. 2004). Electroporation of *Hb9::GFP* into the chick neural tube resulted in GFP expression in post-mitotic motor neurons, whereas Olig2$^+$ pMN cells were unlabeled (Fig. 4C). Coelectroporating *Hb9::GFP* together with *CMV* constructs that drive the constitutive expression of Olig2 blocked GFP expression in post-mitotic motor neurons (Fig. 4D,E). As expected, the inclusion of Olig2 triggered the formation of ectopic GFP$^+$ motor neurons in the V2 domain, supporting the argument against a nonspecific suppression of Hb9 expression by Olig2 (Fig. 4E). In addition, this provides further evidence that the regulatory interactions in the V2 cell domain differ from those in the endogenous motor neuron population. Next we tested whether DNA binding is a prerequisite for Olig2 to suppress *Hb9* transcription. The Olig2 DNA-binding mutant Olig2-AQ was less active at suppressing Hb9::GFP expression than the native Olig2 protein (Fig. 4F), suggesting that Olig2 must bind to the *Hb9* gene to efficiently suppress its expression.

Olig2 interacts with E-box elements to suppress gene expression

To better understand the mechanism by which Olig2 suppresses gene expression in post-mitotic motor neurons, we investigated how the *Hb9* gene was negatively regulated by this bHLH factor. Previous studies have shown that protein complexes consisting of Ngn2:E47 interact with two E-box elements located within the M50 and M100 segments of the *Hb9* enhancer and synergize with DNA-bound complexes of Isl1 and Lhx3 to promote high-level gene expression in post-mitotic motor neurons (Fig. 5A; Lee and Pfaff 2003; Lee et al. 2004). Point mutations within the M50 E-box were found to unmask the repression of the Hb9 promoter in the pMN cell domain, leading to ectopic expression of the GFP reporter in transgenic mice (data not shown) and electroporated chick embryos (Fig. 5A,B). Mutation of the M100 E-box element also resulted in premature expression of GFP in motor neuron progenitors (Fig. 5A). Likewise, combining point mutations in both the M50 and M100 E-box elements dramatically reduced the level of enhancer activity in motor neurons and led to the derepression of the reporter in pMN cells (Fig. 5A). These results suggested that the E-box sequences within the *Hb9* enhancer are bifunctional regulatory elements: acting to repress premature Hb9 expression in Olig2$^+$ pMN cells, and to activate transcription in post-mitotic motor neurons.

Since binding sites for Olig2 have not been defined, we first tested whether Olig2 could interact with the E-box elements within the M50 and M100 regions of the enhancer. Mobility shift experiments detected binding to both E-box elements in a manner that required the intact basic domain of Olig2 (Fig. 5C; data not shown). Competition with unlabeled oligonucleotides containing the native M50 E-box sequence, point mutated E-box, or unrelated sequence showed that only the native E-box se-

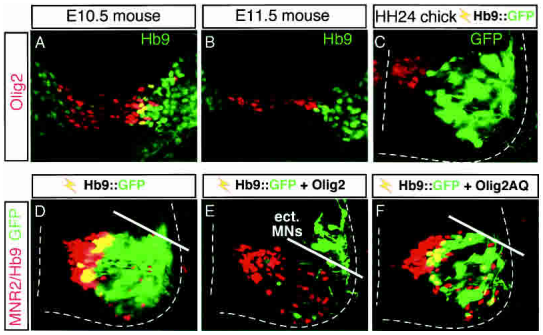

Figure 4. Olig2 represses gene expression in post-mitotic motor neurons. (*A*,*B*) Immunohistochemical analysis of embryonic day 10.5–11.5 (E10.5–E11.5) mouse ventral neural tube with antibodies against Olig2 (red) and the motor neuron marker Hb9 (green). pMN cells located laterally down-regulate Olig2 as they differentiate into Hb9$^+$ motor neurons. In contrast, undifferentiated pMN cells within the ventricular zone maintain Olig2 expression. (*C–F*) GFP labeling driven from a reporter (*Hb9::GFP*) containing the motor neuron enhancer (M250 element, MN^E) isolated from the *Hb9* gene (Lee et al. 2004) in combination with Olig2 or Hb9/MNR2 expression in HH stage 24 chick embryos. MNR2 monoclonal antibody staining detects two Hb9-related proteins in chick, MNR2 in progenitors and young motor neurons, and to a lesser extent Hb9 in mature motor neurons (Tanabe et al. 1998). (*C*) Electroporation of Hb9::GFP results in GFP expression (green) in post-mitotic motor neurons but not Olig2$^+$ (red) pMN cells. (*D*) Electroporation of the Hb9::GFP promoter construct labels only the lateral Hb9/MNR2$^+$ cells (yellow). (*E*) Constitutive expression of Olig2 suppressed Hb9::GFP in post-mitotic motor neurons, but induced GFP expression in the V2 interneuron domain where ectopic motor neurons form (ect. MNs). (*F*) The Olig2-AQ DNA-binding mutant is less efficient at suppressing Hb9::GFP promoter activity in motor neurons and fails to induce ectopic expression of GFP in the V2 domain.

quence could compete for Olig2 binding (Fig. 5D). These results provide strong evidence that Olig2 binds to DNA in a sequence-specific manner. In general, cell-type-restricted bHLH proteins (B class factors) require heterodimerization with more broadly distributed A class bHLH proteins such as E12 and E47 to bind DNA and activate transcription (Henthorn et al. 1990; Hu et al. 1992). We next used antibodies to specific components of the bHLH complexes to determine whether Olig2 partnered with other factors to bind DNA. We found that DNA-bound complexes of Olig2:E47 could be detected by supershift analysis; however, the preferred complex appeared to be generated by Olig2 alone (Fig. 5E). Because Olig2 readily self-associates (see below), the Olig2-alone DNA complexes detected in our binding reactions are likely to represent Olig2:Olig2 complexes.

To establish whether Olig2 could bind to the E-box elements within the *Hb9* promoter in vivo, we used chromatin immunoprecipitation (ChIP) to monitor protein–DNA interactions. Wild-type Olig2 was found to bind the *Hb9* promoter following P19 cell transfection, whereas the DNA-binding mutant Olig2-AQ failed to interact with the *Hb9* gene (Fig. 5F). Next we tested whether mutations in the E-boxes of *Hb9* would disrupt Olig2's interactions with DNA. Indeed, wild-type Olig2 failed to interact with the *Hb9* enhancer containing E-box mutations in the M250 regulatory element (Fig. 5G), consistent with the in vitro binding detected using EMSA. Taken together, our data provide strong evidence that Olig2 binds directly to the E-box elements within the *Hb9* gene.

Olig2 protein–protein interactions

Recent studies have shown that Olig2 binds to the homeodomain transcription factor Nkx2.2 and the Id2/4 helix–loop–helix proteins (Sun et al. 2003; Samanta and Kessler 2004). We further characterized Olig2 interactions with basic helix–loop–helix proteins using yeast two-hybrid and immunoprecipitation assays. The yeast assay detected a strong self-binding between Olig2 proteins, as well as weaker heterointeractions with E47 and Ngn2 (Fig. 6A,B; Samanta and Kessler 2004; data not shown). In contrast, B class bHLH factors such as Ngn2 or NeuroM were unable to self-bind, and only formed heterodimers with A class factors such as E47 (Fig. 6A,B; data not shown). Immunoprecipitations of in vitro expressed proteins were used as an independent measure for examining these protein–protein interactions. Consistent with the results from the yeast two-hybrid analysis, we found that HA-tagged Olig2 interacted strongly with Flag-tagged Olig2 and to a lesser degree with Flag-tagged Ngn2 (Fig. 6C). Conversely, HA-tagged Ngn2 was found to precipitate with Flag-tagged Olig2 and E47 (Fig. 6C). Taken together, the protein interaction and DNA-binding studies suggest that both homo- and heteromeric forms of Olig2 can bind E-box elements, although the homomeric Olig2–Olig2 complex appears to be the preferred species (Fig. 5E).

Olig2 represses transcription

The finding that E-box elements mediate silencing of post-mitotic motor neuron genes in pMN cells, combined with the observation that Olig2 binds to E-box elements, raised the possibility that Olig2 is a direct transcriptional repressor of the *Hb9* gene acting to block its premature expression in the progenitor cells for motor neurons and oligodendrocytes. To test this hypothesis, 293 cells were transfected with luciferase reporters and Olig2-expression constructs. The native Olig2 protein strongly repressed *Hb9*-enhancer activity, whereas the DNA-binding mutant Olig2-AQ failed to repress transcription (Fig. 7A). Mutation of the E-boxes within the motor neuron enhancer attenuated the ability of Olig2 to repress *Hb9* transcription (Supplementary Fig. S1A), in agreement with the finding that these mutations disrupt Olig2–DNA binding. Taken together, these findings indicate that Olig2 is a direct repressor of the *Hb9* gene.

Since the activator Ngn2 and repressor Olig2 are co-expressed in pMN cells and both bind to E-box elements,

we examined whether a competitive relationship might exist between these two types of transcription factors. Ngn2 stimulated transcription from an E-box:luciferase reporter in both 293 and P19 cells, as expected (Fig. 7B; Supplementary Fig. S1B). The addition of Olig2, however, interfered with the activation of the E-box reporter by Ngn2 (Fig. 7B; Supplementary Fig. S1B). In principle, the inhibitory effect of Olig2 on transcription could be based on a mechanism that requires DNA binding and/or protein–protein interactions independent from DNA binding. To test which mechanism(s) accounts for Olig2-mediated repression, we examined whether DNA-binding mutants of Olig2 (Olig2-AQ) could suppress Ngn2-mediated activation via potential protein interactions that might squelch the activity of Ngn2:E47 complexes. We found that Olig2-AQ was partially, but not fully, able to suppress Ngn2-mediated activation (Supplementary Fig. S1B), suggesting that DNA binding contributes to repression.

Previous studies have firmly established that Olig2 functions as a dedicated transcriptional repressor in order to specify motor neuron and oligodendrocyte cell fate (Mizuguchi et al. 2001; Novitch et al. 2001; Qi et al. 2001). Gal4-based assays have demonstrated that Olig2 can recruit corepressors in Cos-1 cells (Novitch et al. 2001), but the ability of native Olig2 to recruit corepressors in the neural tube to repress transcription was unknown. We coelectroporated Gal4 alone, Gal4–Olig2, and Gal4–Ngn2 together with a UAS-LacZ reporter and monitored whether LacZ expression was repressed or activated. The UAS reporter was expressed throughout the dorsal–ventral axis of the neural tube in the presence of Gal4 alone, and expression was enhanced by Gal4–Ngn2 (Fig. 7C–E,I–K). In contrast, the reporter was suppressed by Gal4–Olig2 fusions (Fig. 7F–H). These studies indicate that Olig2 can recruit corepressors that are widely avail-

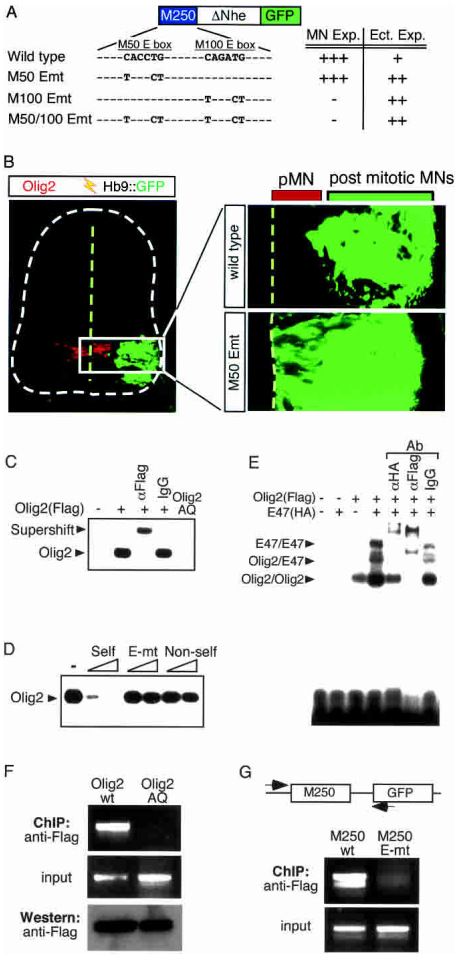

Figure 5. Olig2 interacts with bifunctional activator/suppressor E-boxes within *Hb9*. (*A*) Schematic representation of the M250 motor neuron enhancer element isolated from the *Hb9* gene linked to the proximal region of the *Hb9* promoter (ΔNhe). Two evolutionarily conserved E-boxes are located within the enhancer (M50 E-box and M100 E-box) (Lee et al. 2004). Wild-type and E-box mutants were electroporated into chick embryos, and the relative level of GFP in motor neurons (MN Exp.) and ectopic sites (Ect. Exp.) was monitored at HH stage 24. The table is representative of ~10 embryos for each construct (+++ indicates strong expression; ++ indicates moderate expression; + indicates low-level expression). (*B*) The wild-type M250 enhancer in the Hb9::GFP reporter is inactive in the pMN cell domain marked by Olig2 expression (red). In contrast, mutation of the M50 E-box element within M250 results in the derepression of Hb9 expression in the progenitor cell domain. Likewise, mutation of the M100 E-box, and M50 and M100 E-boxes in combination, also led to ectopic expression in the pMN domain (*A*; data not shown). (*C*,*D*) Gel retardation assays using the M100A probe containing the M100 E-box from the *Hb9* enhancer (Lee et al. 2004). (*C*) Olig2 binds to the M100A oligonucleotide, whereas the Olig2 DNA-binding mutant Olig2-AQ lacks this activity. The M100A DNA–Olig2 protein complex was supershifted by antibody against the Flag epitope tag, but not by control IgG antibody. Probes for the M50 E-box element exhibit the same pattern of Olig2 binding (data not shown). (*D*) The M100A DNA–Olig2 protein interaction was challenged by 20- or 100-fold molar excess of the unlabeled M100A probe (Self), E-box mutated M100A (E-mt), or unrelated oligo (Non-self). Olig2 interacts with DNA E-box elements in a sequence-specific manner. (*E*) A presumed Olig2/Olig2 homodimer and Olig2/E47 heterodimer bind to the E-box site within the M100A probe. Similar results were obtained with the E-box site within the M50 probe (data not shown). Flag-tagged Olig2 and HA-tagged E47 were translated in vitro separately and incubated with the M100A DNA. Supershift assays using antibodies against the HA or Flag epitope tags confirmed the protein composition in each complex. (*F*,*G*) Chromatin immunoprecipitation experiments in transfected P19 cells. (*F*) Flag-tagged Olig2 wt (wild type) is detected bound to the endogenous *Hb9* promoter, whereas the DNA-binding mutant Olig2-AQ failed to interact with this DNA sequence. Western blot analysis detects similar levels of protein expression. (*G*) Flag-tagged Olig2 wt was transfected into P19 cells with two derivatives of the *Hb9* promoter: one carrying the native motor neuron enhancer (M250 wt) and the other with mutated E-box elements (M250 Emt). Primers that were selective for the transfected *Hb9* promoter construct were used as shown in the schematic. Olig2 failed to bind to the *Hb9* promoter with mutated E-box elements.

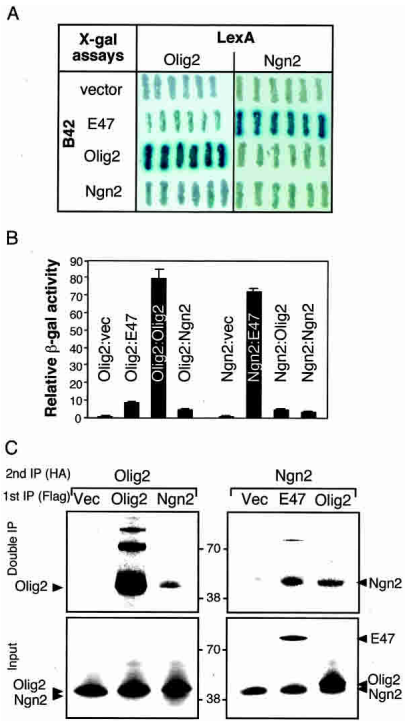

Figure 6. Olig2 self-associates and forms weak heterointeractions with E47 and Ngn2. (*A*,*B*) Protein interactions between bHLH factors assayed using the yeast two-hybrid assay. Olig2 protein interacts strongly with itself, and weakly with E47 and Ngn2. Ngn2 does not self-associate but forms high-affinity heterodimers with E47. (*C*) In vitro pull-down assays using Flag and HA epitope tags for immunoprecipitation. Proteins were co-translated with ^{35}S-labeled methionine, then complexes were isolated with Flag antibody, dissociated, and HA-proteins were identified by immunopurification and electrophoresis. Olig2 associates strongly with itself. Olig2 and Ngn2 appear to interact with lower affinity.

able within the neural tube to actively silence gene expression.

Discussion

Spinal pMN cells represent a discrete subpopulation of bipotential neuroepithelial precursors that produce motor neurons and oligodendrocytes (Richardson et al. 2000; Briscoe and Ericson 2001). These progenitor cells are marked by a unique combination of bHLH and homeodomain transcription factors that become serially activated as these cells develop (Briscoe et al. 2000; Mizuguchi et al. 2001; Novitch et al. 2001; Zhou et al. 2001). Since the mechanisms that direct the fate of pMN cells toward a neuronal versus a glial identity are poorly understood, we examined how gene regulation is controlled at the transition point when these progenitor cells generate motor neuron progeny. We provide evidence that the bHLH transcription factor Olig2 functions as a pMN "maintenance" factor that blocks gene expression for post-mitotic motor neuron formation. This function appears to represent a control measure for preventing the overproduction of motor neurons at the expense of precursors for oligodendrocytes. Below we discuss how the regulatory interactions between Olig2

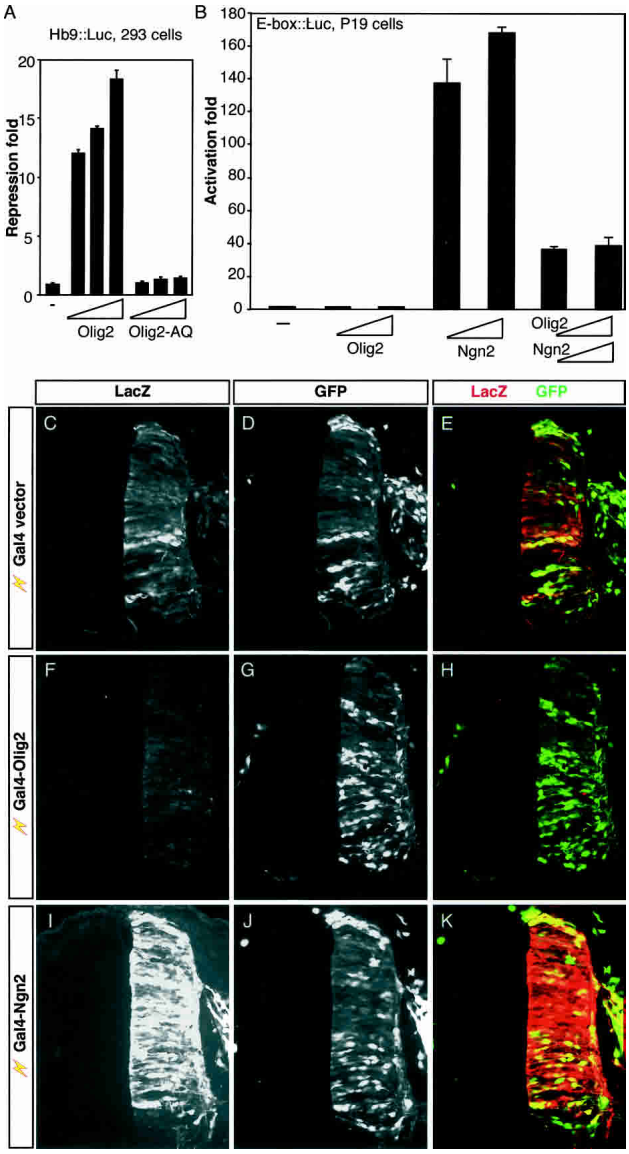

Figure 7. Olig2 and Ngn2 function antagonistically to control gene expression. (*A*) Olig2 strongly inhibited luciferase expression in 293 cells driven from the Hb9 motor neuron enhancer construct *Hb9::Luc*. The Olig2-AQ DNA-binding mutant was not able to repress luciferase expression. (*B*) Olig2 suppressed Ngn2-mediated *trans*-activation of an E-box reporter in 293 cells. (*C–K*) In vivo Gal4-based assays to measure corepressor function with a UAS-LacZ construct in HH stage 24 electroporated chick embryo neural tubes. CMV-GFP is included as an independent electroporation control in the assays. (*C–E*) Gal4 alone does not suppress LacZ expression. (*F–H*) The Gal4–Olig2 fusion strongly inhibits LacZ expression along the entire dorsal–ventral axis of the neural tube, but does not interfere with GFP. (*I–K*) Gal4–Ngn2 has the opposite effect of Olig2 and stimulates LacZ expression.

and Ngn2 are used to form a molecular switch for controlling the development of pMN cells.

A bHLH network for directing the fate of pMN cells

An early step in the process of cell fate specification in the neural tube is the establishment of progenitor cell populations allocated for the production of specific progeny (Briscoe et al. 2000; Jessell 2000). This phase of development is associated with the expression of unique combinations of bHLH and homeodomain transcription factors involved in setting the proper patterns of gene expression for controlling cell identity. pMN cells marked by Olig2, however, appear to retain a certain degree of plasticity during early stages of neural tube development (Richardson et al. 2000). For example, cell marking experiments in zebrafish show that secondary motor neurons and oligodendrocyte precursors arise from a common progenitor pool (Park et al. 2004). The transcriptional code that sets pMN cells apart from neighboring neuroepithelial cells is established sequentially and is first comprised of the homeodomain proteins Pax6 and Nkx6.1, followed shortly thereafter by the appearance of Olig2 (Fig. 8A; Briscoe et al. 2000; Novitch et al. 2001). Olig2 represents a key regulator of pMN development because it primes these precursors to become motor neurons by coordinately activating bHLH factors for neurogenesis and homeodomain factors for neuronal subtype specification (Novitch et al. 2001).

Our studies suggest that Olig2 not only activates the expression of transcriptional pathways for motor neuron specification but also antagonizes the further differentiation of pMN cells by repressing the expression of genes such as *Hb9* involved in post-mitotic motor neuron differentiation. Thus, Olig2 functions to first establish and then maintain the transient pMN cell population in the neural tube. The antagonistic function of Olig2 on the further development of pMN cells may explain why misexpression studies have uncovered a dichotomy between its ability to activate genes such as Ngn2 and Lhx3 that contribute to motor neuron formation and its ability to actually trigger the formation of ectopic post-mitotic motor neurons (Mizuguchi et al. 2001; Novitch et al. 2001). Although the blockade of post-mitotic motor neuron differentiation by Olig2 is well suited to preserve pMN cells into later stages of development for oligodendrocyte formation, it raises the question of how motor neurons are produced when their progenitors express this inhibitory factor. We found that Ngn2 functions as a counterbalance to Olig2. This finding combined with the observation that Olig2 promotes the expression of Ngn2 (Mizuguchi et al. 2001; Novitch et al. 2001; Lu et al. 2002; Zhou and Anderson 2002) provides evidence that a negative-feedback loop is operative in developing pMN cells based on a network of bHLH factors.

Examination of the bHLH profiles in pMN cells indicates that they express heterogeneous levels of Olig2 and Ngn2. The variation among individual cells in their levels of these bHLH factors might serve as the basis for allowing a subset of pMN cells to differentiate into motor neurons while preserving some of the progenitors for oligodendrocyte formation. Cells located laterally in the subventricular zone express the highest levels of Ngn2 relative to Olig2, whereas the most medial cells contain higher levels of Olig2 compared to Ngn2. As motor neurons emerge from the ventricular zone they extinguish their expression of Olig2, whereas Ngn2 is down-regulated in pMN cells as development progresses and oligo-

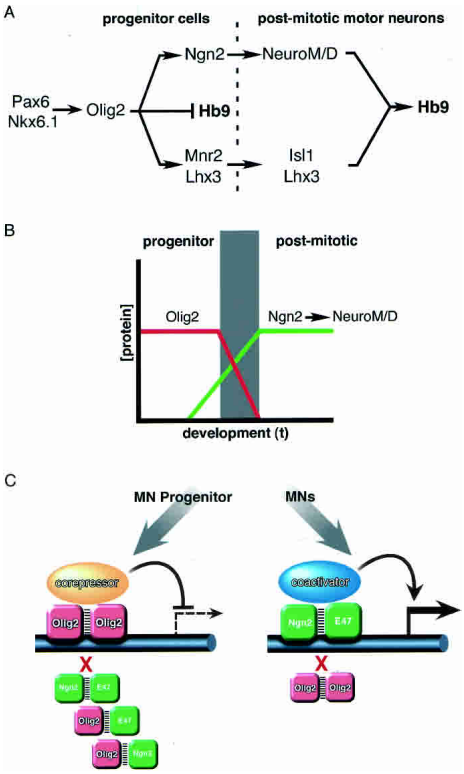

Figure 8. Model of Olig2 and Ngn2 function in motor neuron specification. (*A*) A transcriptional cascade initiated by Shh and RA leads to the expression of Olig2 in pMN cells that serve as the precursors for motor neurons and oligodendrocytes. Olig2 occupies a key nodal point in the pathway, contributing to the regulation of homeodomain factors for motor neuron subtype specification (Mnr2, Lhx3, Isl1), and bHLH factors (Ngn2, NeuroM/D) for neurogenesis and synergistic cooperation with the homeodomain factors. Here we provide evidence that a key function of Olig2 is to maintain the progenitor cell state by repressing the expression of genes such as Hb9 found in post-mitotic motor neurons. (*B*) Olig2 and Ngn2 function antagonistically to either suppress or activate motor neuron gene expression, respectively. The fate decision of pMN cells to become post-mitotic motor neurons or remain in reserve for the later formation of oligodendrocytes is thought to depend on the relative levels of the repressor Olig2 to the activators Ngn2 and NeuroM/D. (*C*) Olig2 uses both DNA-dependent and DNA-independent mechanisms to suppress motor neuron gene expression in pMN cells. Protein–protein interactions between Olig2 and the activator proteins Ngn2 and E47 are likely to squelch Ngn2:E47 dimer formation and minimize their ability to bind and activate gene expression. In addition, the binding sites for Olig2 complexes and Ngn2 complexes overlap, suggesting they compete for their DNA targets.

dendrocyte formation is initiated. Although Olig2 is found to trigger the expression of Ngn2, the expression of Ngn2 is likely fine tuned by integrating multiple regulatory inputs (Scardigli et al. 2001). For example, the notch–delta pathway plays a prominent role in controlling the cell-to-cell patterns of proneural bHLH factor expression (Bertrand et al. 2002). Zebrafish studies have shown that Notch-signaling-deficient embryos express elevated levels of Ngn and generate an excess of motor neurons at the expense of oligodendrocyte precursors (Appel and Eisen 1998; Park and Appel 2003). Conversely, enhanced notch signaling suppresses Ngn expression in Olig2$^+$ pMN cells favoring oligodendrocyte development (Park and Appel 2003). These observations combined with the findings in this report support a model in which motor neuron differentiation is gated by the relative levels of Ngn and Olig bHLH proteins in pMN cells (Fig. 8B; Park and Appel 2003). Thus, progenitors with Olig2^{high}/Ngn^{low} are predicted to serve as progenitors for oligodendrocytes, whereas pMNs with Olig2^{low}/Ngn2^{high} are more likely to produce motor neurons.

Gene regulation in spinal progenitor cells

The mechanistic underpinnings of Olig2's activities in pMN cells have previously been difficult to characterize due to the lack of bona fide gene targets for this transcription factor. The E-box elements within the motor neuron enhancer of the *Hb9* gene interact with Ngn2 and NeuroM/D, which promote high-level transcription of this homeodomain transcription factor involved in post-mitotic motor neuron development (Lee and Pfaff 2003; Lee et al. 2004). We found that point mutations within the E-box elements cause premature transcription of *Hb9* in pMN progenitors due to the derepression of general activators bound to the proximal promoter (Lee et al. 2004), which are capable of driving widespread transcription of the *Hb9* gene. Thus, E-box elements serve a dual function in motor neuron development: to repress expression in progenitors and to activate transcription in post-mitotic cells. We found that Olig2 binds in a sequence specific manner to the canonical E-box elements within *Hb9*, but unlike other bHLH factors the binding was not dependent on a heterodimerization protein partner. Biochemical studies indicate that Olig2 can bind to itself with high affinity, and to a lesser extent with A class bHLH proteins such as E47 (Samanta and Kessler 2004) and B class partners such as Ngn2. The strong self-dimerization of Olig2 combined with the ability of Olig2 to bind to E-box elements in the absence of heterodimerization partners suggests that Olig2 binds DNA predominantly as a homodimer.

Based on the DNA- and protein-binding interactions we have observed, Olig2 appears to use a combination of passive and direct mechanisms for silencing post-mitotic motor neuron gene expression (Fig. 8C). The protein–protein interactions of Olig2 indicate that it can bind to both E47 and Ngn2. This raised the possibility that Olig2 might be able to squelch the activity of these proteins in a manner that is analogous to the negative actions of the Id proteins on the proneural bHLH factors (Norton 2000). A prediction of this model is that DNA-binding mutants of Olig2 (Olig2-AQ) should retain the ability to block Ngn2:E47 activity. We found that Olig2-AQ could slightly attenuate the activity of Ngn2:E47 in transcription assays; however, this mutant form of Olig2 was much less active than the native protein at suppressing *Hb9* reporters in post-mitotic motor neurons. These findings suggest that negative-acting protein–protein interactions probably account for only part of Olig2's repressor function. DNA-binding studies showed that Olig2 interacts with E-box elements shared by Ngn2:E47. Thus, Olig2 binding to DNA likely forces the passive displacement of activator complexes comprised of proneural bHLH proteins from DNA targets. In addition, Gal4–Olig2 fusions demonstrate that Olig2 recruits corepressors that act in a dominant fashion to repress transcription (Novitch et al. 2001). Thus, E-box elements represent key sites for integrating both positive and negative inputs for gene expression in motor neurons and consequently serve an important role in controlling the timing of gene expression.

The putative corepressors used by Olig2 to suppress transcription have not been well characterized. Previous studies have shown that the fusion of the groucho interaction domain from engrailed to the DNA-binding domain of Olig2 is sufficient to reconstitute its activity in vivo (Mizuguchi et al. 2001; Novitch et al. 2001; Zhou et al. 2001). Although Gro/TLE corepressors play a prominent role in neural tube patterning (Muhr et al. 2001), it remains unclear whether Olig2 normally interacts with these corepressors or uses another family of proteins such as the CtBPs to repress transcription. Nevertheless, our in vivo analysis of Gal4–Olig2 repressor activity indicates that Olig2 is able to recruit corepressors within a broad population of cell types along the dorsal–ventral axis of the neural tube. Furthermore, the wide distribution of Olig2 corepressor proteins suggests that these factors do not account for the limited ability of Olig2 to trigger motor neuron differentiation when ectopically expressed in the neural tube.

Motor neuron specification by Olig2

Our findings indicate that constitutive expression of Olig2 is antagonistic to motor neuron differentiation; however, one notable exception to this effect is observed in the V2 interneuron domain of the spinal cord. Here, vectors constitutively expressing Olig2 are able to trigger ectopic motor neuron formation (Mizuguchi et al. 2001; Novitch et al. 2001) and likewise fail to block *Hb9* reporter expression (see Fig. 4E). The mechanistic basis for this difference in the activity of Olig2 within the V2 interneuron domain is unknown, but could reside in the precise temporal and spatial patterns and levels of other bHLH factors that are likely to influence the function of Olig2 through protein–protein interactions and competition for DNA binding. For example, inclusion of Ngn2 with Olig2 appears to enhance the ability of Olig2 to

promote ectopic motor neuron differentiation in the hindbrain (Mizuguchi et al. 2001). Regardless of the mechanism, however, relieving the inhibitory block of Olig2 on post-mitotic motor neuron gene expression correlates with its ability to trigger motor neuron formation.

Our results and those of others suggest that Olig2 contributes to the establishment of a pMN cell identity in two ways: it acts to prime cells for differentiation by derepressing (i.e., indirectly activating) genes for cell fate specification and neurogenesis such as *Lhx3* and *Ngn2* (Mizuguchi et al. 2001; Novitch et al. 2001; Lu et al. 2002; Zhou and Anderson 2002), while at the same time preventing their further differentiation. Our findings suggest that an obligatory step for differentiation of the endogenous motor neuron population is the down-regulation of Olig2, in order to release the repressive block of this factor on neurogenesis and post-mitotic motor neuron gene expression. Consequently, the use of constitutive drivers of Olig2 for misexpression are likely to generate ectopic pMN progenitor cells but obstruct their further differentiation into motor neurons. By extension, applications using Olig2 to convert stem cells into motor neurons for treating diseases are likely to work more efficiently if Olig2 is expressed transiently rather than constitutively.

Materials and methods

DNA constructs

Mouse Olig2, Ngn2, and E47 were subcloned into pCS2 (Turner and Weintraub 1994), pcDNA3 (Invitrogen) containing a HA or Flag tag, pMES, pIRES2-EGFP (Clontech), pM (Clontech) encoding the Gal4 DNA-binding domain, and LexA- and B42-vectors (Gyuris et al. 1993) for yeast two-hybrid assays. Olig2-AQ point mutant (Olig2 KR 119,120 AQ) was generated using a PCR-based mutagenesis method (QuickChange; Stratagene). *UAS-tk-LacZ* reporter (gift of R. Evans, Salk Institute, La Jolla, CA) and pEGFP (Clontech) were used for in vivo Gal4-based transcription assays. Isl1 and Lhx3 expressed from the pCS2 vector and reporter constructs *Hb9::GFP*, *Hb9::Luciferase*, and *E-box::Luciferase* have been described previously (Thaler et al. 2002; Lee and Pfaff 2003; Lee et al. 2004).

In ovo electroporation

Chick embryos (SPAFAS, McIntyre Farms) were incubated in a humidified chamber and staged according to Hamburger and Hamilton (HH) (Hamburger and Hamilton 1951). DNA constructs were injected into the lumens of HH stage 12–13 chick embryonic spinal cords. Electroporation was performed using a square wave electroporator (BTX) as described previously (Itasaki et al. 1999; Nakamura et al. 2000). Incubated chicks were harvested and analyzed at HH stage 18–25.

Immunocytochemistry

Immunohistochemistry was performed as described previously (Thaler et al. 1999) using the following antibodies: mouse anti-MNR2/HB9 (5C10) (Tanabe et al. 1998), rabbit anti-Hb9 (Thaler et al. 1999), rabbit anti-Isl1 (Ericson et al. 1992), rabbit anti-LacZ (Sigma), rabbit or guinea pig anti-Olig2 (Mizuguchi et al. 2001; Novitch et al. 2001), rabbit anti-Ngn2 (Zhou et al. 2001), and mouse anti-β tubulin TuJ1 (BabCo).

Cell culture and transient transfection assays

293 and P19 cells were cultured in Dulbecco's modified Eagle medium or α-minimum essential medium supplemented with 10% bovine fetal serum. Cells were seeded into 48-well plates and transient transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. A CMV-β-galactosidase plasmid was cotransfected for normalization of transfection efficiency, and empty vectors were used to equalize the total amount of DNA. Cells were harvested 36 h after transfection. Cell extracts were assayed for luciferase activity and the values were corrected with β-galactosidase activity. Data are represented as means of triplicate values obtained from representative experiments. All transfections were repeated at least three times. For motor neuron induction assays, P19 mouse embryonic cells were transfected, and cultured for additional 2–3 d, fixed, and immunostained with motor neuron marker anti-Hb9 antibody or pan-neuronal marker anti-β tubulin TuJ1 for analysis.

Electrophoretic mobility shift assays (EMSA) and chromatin immunoprecipitation (ChIP)

EMSA was performed as previously described (Lee and Pfaff 2003). The sense sequence of M50 and M100A is shown below. M50, 5′-GGAGTGGGCCACCTGTTCCTTTGGGTAATTATTTTAATGTAAGGGG; M100A, 5′-CAGCCTTTCAGTGGAATTTCCCAGATGGGCCAAGGGGAGGCCAGG. The E-box sequence in each oligo is underlined. ChIP experiments were performed with P19 cells harvested 24 h after transfection with Olig2-Flag-tagged expression constructs and cross-linked with 1% formaldehyde for 10 min. The nuclear fraction was isolated and sonicated to shear genomic chromatin, clarified by centrifugation, precleared with protein A beads, and immunoprecipitated with anti-Flag antibody (Eastman Kodak) or control IgG (Santa Cruz Biotechnology). Following immunoprecipitation and immobilization of immunocomplexes, proteinase K digestion was allowed to proceed at 65°C overnight. DNA was purified using QIAquick spin columns (QIAGEN). PCR was carried out on the eluted DNA using primers to the endogenous mouse *Hb9* gene or primers specific to transfected *Hb9* promoter constructs containing native or mutated E-box sequences.

Yeast two-hybrid assays

The cotransformation, X-gal assays, and quantitative liquid β-galactosidase assays in yeast were performed as described previously (Lee et al. 1998). For each experiment, at least three independently derived colonies were tested.

In vitro immunoprecipitation assays

[^{35}S]methionine-labeled Olig2, Ngn2, and E47 proteins were produced using TNT reticulocyte lysate (Promega). Anti-Flag M2 antibody (Kodak) and anti-HA antibody (BabCo) were used for immunoprecipitation. Coimmunoprecipitation was performed as previously described (Lee and Pfaff 2003).

Acknowledgments

We are grateful to H. Juguilon in the R. Evans laboratory for assistance with transfections. C. Stiles and H. Takebayashi

kindly provided clones of Olig1 and Olig2. Antibodies to detect Olig2 were generous gifts from M. Nakafuku and T. Jessell, and D. Anderson kindly provided Ngn2 antibody. We thank G. Gill, C. Kintner, and B. Novitch for advice and insightful comments on the manuscript. S.-K.L. was supported by a postdoctoral fellowship from the Human Frontiers Science Program, and S.L.P. was supported by the Helen McLoraine Developmental Chair in Neurobiology and the G. Harold and Leila Y. Mathers Foundation. This research was supported by NINDS grant RO1NS37116.

References

Appel, B. and Eisen, J.S. 1998. Regulation of neuronal specification in the zebrafish spinal cord by Delta function. *Development* **125:** 371–380.

Arber, S., Han, B., Mendelsohn, M., Smith, M., Jessell, T.M., and Sockanathan, S. 1999. Requirement for the homeobox gene Hb9 in the consolidation of motor neuron identity. *Neuron* **23:** 659–674.

Bertrand, N., Castro, D.S., and Guillemot, F. 2002. Proneural genes and the specification of neural cell types. *Nat. Rev. Neurosci.* **3:** 517–530.

Briscoe, J. and Ericson, J. 2001. Specification of neuronal fates in the ventral neural tube. *Curr. Opin. Neurobiol.* **11:** 43–49.

Briscoe, J., Pierani, A., Jessell, T.M., and Ericson, J. 2000. A homeodomain protein code specifies progenitor cell identity and neuronal fate in the ventral neural tube. *Cell* **101:** 435–445.

Cepko, C.L. 1999. The roles of intrinsic and extrinsic cues and bHLH genes in the determination of retinal cell fates. *Curr. Opin. Neurobiol.* **9:** 37–46.

Ericson, J., Thor, S., Edlund, T., Jessell, T.M., and Yamada, T. 1992. Early stages of motor neuron differentiation revealed by expression of homeobox gene Islet-1. *Science* **256:** 1555–1560.

Ericson, J., Rashbass, P., Schedl, A., Brenner-Morton, S., Kawakami, A., van Heyningen, V., Jessell, T.M., and Briscoe, J. 1997. Pax6 controls progenitor cell identity and neuronal fate in response to graded Shh signaling. *Cell* **90:** 169–180.

Farah, M.H., Olson, J.M., Sucic, H.B., Hume, R.I., Tapscott, S.J., and Turner, D.L. 2000. Generation of neurons by transient expression of neural bHLH proteins in mammalian cells. *Development* **127:** 693–702.

Gyuris, J., Golemis, E., Chertkov, H., and Brent, R. 1993. Cdi1, a human G1 and S phase protein phosphatase that associates with Cdk2. *Cell* **75:** 791–803.

Hamburger, V. and Hamilton, H. 1951. A series of normal stages in the development of chick embryo. *J. Morphol.* **88:** 49–92.

Henthorn, P., Kiledjian, M., and Kadesch, T. 1990. Two distinct transcription factors that bind the immunoglobulin enhancer microE5/κ 2 motif. *Science* **247:** 467–470.

Hu, J.S., Olson, E.N., and Kingston, R.E. 1992. HEB, a helix–loop–helix protein related to E2A and ITF2 that can modulate the DNA-binding ability of myogenic regulatory factors. *Mol. Cell. Biol.* **12:** 1031–1042.

Itasaki, N., Bel-Vialar, S., and Krumlauf, R. 1999. 'Shocking' developments in chick embryology: Electroporation and in ovo gene expression. *Nat. Cell Biol.* **1:** E203–E207.

Jessell, T.M. 2000. Neuronal specification in the spinal cord: Inductive signals and transcriptional codes. *Nat. Rev. Genet.* **1:** 20–29.

Lee, S.K. and Pfaff, S.L. 2003. Synchronization of neurogenesis and motor neuron specification by direct coupling of bHLH and homeodomain transcription factors. *Neuron* **38:** 731–745.

Lee, S.K., Na, S.Y., Kim, H.J., Soh, J., Choi, H.S., and Lee, J.W. 1998. Identification of critical residues for heterodimerization within the ligand-binding domain of retinoid X receptor. *Mol. Endocrinol.* **12:** 325–332.

Lee, S.K., Jurata, L.W., Funahashi, J., Ruiz, E.C., and Pfaff, S.L. 2004. Analysis of embryonic motoneuron gene regulation: Derepression of general activators function in concert with enhancer factors. *Development* **131:** 3295–3306.

Liu, Y. and Rao, M.S. 2004. Olig genes are expressed in a heterogeneous population of precursor cells in the developing spinal cord. *Glia* **45:** 67–74.

Lu, Q.R., Yuk, D., Alberta, J.A., Zhu, Z., Pawlitzky, I., Chan, J., McMahon, A.P., Stiles, C.D., and Rowitch, D.H. 2000. Sonic hedgehog—Regulated oligodendrocyte lineage genes encoding bHLH proteins in the mammalian central nervous system. *Neuron* **25:** 317–329.

Lu, Q.R., Sun, T., Zhu, Z., Ma, N., Garcia, M., Stiles, C.D., and Rowitch, D.H. 2002. Common developmental requirement for Olig function indicates a motor neuron/oligodendrocyte connection. *Cell* **109:** 75–86.

Marquardt, T. and Pfaff, S.L. 2001. Cracking the transcriptional code for cell specification in the neural tube. *Cell* **106:** 651–654.

McConnell, S.K. 1995. Constructing the cerebral cortex: Neurogenesis and fate determination. *Neuron* **15:** 761–768.

Mizuguchi, R., Sugimori, M., Takebayashi, H., Kosako, H., Nagao, M., Yoshida, S., Nabeshima, Y., Shimamura, K., and Nakafuku, M. 2001. Combinatorial roles of olig2 and neurogenin2 in the coordinated induction of pan-neuronal and subtype-specific properties of motoneurons. *Neuron* **31:** 757–771.

Muhr, J., Andersson, E., Persson, M., and Jessell, T.M. 2001. Groucho-mediated transcriptional repression establishes progenitor cell pattern and neuronal fate in the ventral neural tube. *Cell* **104:** 861–873.

Nakamura, H., Watanabe, Y., and Funahashi, J. 2000. Misexpression of genes in brain vesicles by in ovo electroporation. *Dev. Growth Differ.* **42:** 199–201.

Norton, J.D. 2000. ID helix–loop–helix proteins in cell growth, differentiation and tumorigenesis. *J. Cell Sci.* **113:** 3897–3905.

Novitch, B.G., Chen, A.I., and Jessell, T.M. 2001. Coordinate regulation of motor neuron subtype identity and pan-neuronal properties by the bHLH repressor Olig2. *Neuron* **31:** 773–789.

Novitch, B.G., Wichterle, H., Jessell, T.M., and Sockanathan, S. 2003. A requirement for retinoic acid-mediated transcriptional activation in ventral neural patterning and motor neuron specification. *Neuron* **40:** 81–95.

Park, H.C. and Appel, B. 2003. Delta–Notch signaling regulates oligodendrocyte specification. *Development* **130:** 3747–3755.

Park, H.C., Shin, J., and Appel, B. 2004. Spatial and temporal regulation of ventral spinal cord precursor specification by Hedgehog signaling. *Development* **131:** 5959–5969.

Qi, Y., Cai, J., Wu, Y., Wu, R., Lee, J., Fu, H., Rao, M., Sussel, L., Rubenstein, J., and Qiu, M. 2001. Control of oligodendrocyte differentiation by the Nkx2.2 homeodomain transcription factor. *Development* **128:** 2723–2733.

Richardson, W.D., Smith, H.K., Sun, T., Pringle, N.P., Hall, A., and Woodruff, R. 2000. Oligodendrocyte lineage and the motor neuron connection. *Glia* **29:** 136–142.

Rowitch, D.H., Lu, Q.R., Kessaris, N., and Richardson, W.D. 2002. An 'oligarchy' rules neural development. *Trends Neurosci.* **25:** 417–422.

Samanta, J. and Kessler, J.A. 2004. Interactions between ID and OLIG proteins mediate the inhibitory effects of BMP4 on oligodendroglial differentiation. *Development* **131:** 4131–4142.

Scardigli, R., Schuurmans, C., Gradwohl, G., and Guillemot, F. 2001. Crossregulation between Neurogenin2 and pathways specifying neuronal identity in the spinal cord. *Neuron* **31:** 203–217.

Sun, Y., Nadal-Vicens, M., Misono, S., Lin, M.Z., Zubiaga, A., Hua, X., Fan, G., and Greenberg, M.E. 2001. Neurogenin promotes neurogenesis and inhibits glial differentiation by independent mechanisms. *Cell* **104:** 365–376.

Sun, T., Dong, H., Wu, L., Kane, M., Rowitch, D.H., and Stiles, C.D. 2003. Cross-repressive interaction of the Olig2 and Nkx2.2 transcription factors in developing neural tube associated with formation of a specific physical complex. *J. Neurosci.* **23:** 9547–9556.

Tanabe, Y. and Jessell, T.M. 1996. Diversity and pattern in the developing spinal cord. *Science* **274:** 1115–1122.

Tanabe, Y., William, C., and Jessell, T.M. 1998. Specification of motor neuron identity by the MNR2 homeodomain protein. *Cell* **95:** 67–80.

Thaler, J., Harrison, K., Sharma, K., Lettieri, K., Kehrl, J., and Pfaff, S.L. 1999. Active suppression of interneuron programs within developing motor neurons revealed by analysis of homeodomain factor HB9. *Neuron* **23:** 675–687.

Thaler, J.P., Lee, S.K., Jurata, L.W., Gill, G.N., and Pfaff, S.L. 2002. LIM factor Lhx3 contributes to the specification of motor neuron and interneuron identity through cell-type-specific protein–protein interactions. *Cell* **110:** 237–249.

Turner, D.L. and Weintraub, H. 1994. Expression of achaete-scute homolog 3 in *Xenopus* embryos converts ectodermal cells to a neural fate. *Genes & Dev.* **8:** 1434–1447.

Zhou, Q. and Anderson, D.J. 2002. The bHLH transcription factors OLIG2 and OLIG1 couple neuronal and glial subtype specification. *Cell* **109:** 61–73.

Zhou, Q., Wang, S., and Anderson, D.J. 2000. Identification of a novel family of oligodendrocyte lineage-specific basic helix–loop–helix transcription factors. *Neuron* **25:** 331–343.

Zhou, Q., Choi, G., and Anderson, D.J. 2001. The bHLH transcription factor Olig2 promotes oligodendrocyte differentiation in collaboration with Nkx2.2. *Neuron* **31:** 791–807.